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Analysis of the Site Occupancy Constraints of Primary Amino Acid Sequences in the Motif Directing Palmitylation of the Vaccinia Virus 37-kDa Envelope Protein

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Vaccinia virus (VV) encodes a 37-kDa envelope protein (p37) that is palmitylated on cysteine residues 185 and 186 of the 372-amino acid protein. We have previously reported on a loosely conserved consensus motif. Further analysis has identified a conserved consensus sequence, Hydro**AAC(C)A* (Hydro* represents a hydrophobic portion of a protein determined by any one of the following: a hydrophobic sequence, a transmembrane domain 1–12 amino acids away from the modification site, or the prior addition of a hydrophobic molecule; C, palmitate acceptor cysteines; *A,* aliphatic residue) that is responsible for directing palmitylation of certain classes of palmitylproteins. We have analyzed the amino acid site occupancy upstream and downstream of the palmitate acceptor residues in p37 by site-directed mutagenesis and transient expression of mutated proteins in VV-infected cells. The two aliphatic alanines naturally found at positions 183 and 184 of the wild-type p37 allow for efficient palmitylation. In contrast, the replacement of leucine at position 187 with glycine increases palmitylation efficiency. The 10 amino acids immediately upstream of the palmitate acceptor site are absolutely necessary while the downstream 10 amino acids are dispensable. These results together with previous data suggests that the Hydro**AAC(C)A* motif is required for efficient palmitylation of p37.

INTRODUCTION

Vaccinia virus (VV),[2] belonging to the *Poxviridae* family of animal viruses, is a large enveloped virus that replicates entirely within the cytoplasm of an infected cell. The double-stranded DNA, 191-kb pair genome has been completely sequenced and encodes ~200 gene products, many of which are responsible for virus replication and propagation in an infected cell (Goebel *et al.,* 1990). Several of these viral polypeptides are known to be either posttranslationally or cotranslationally modified by addition of palmitate or myristate respectively (Child and Hruby, 1992).

The VV replication cycle is a rather complex series of events and is still not fully understood. Infectious virions can exist in one of four states: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV), and extracellular enveloped virus (EEV). IMV, containing the virus core particle, is thought to be formed by budding through the intermediate compartment (Sodeik *et al.,* 1993), a cellular structure located between the endoplasmic reticulum and the Golgi, resulting in the acquisition of a double-layered membrane. The IMV particle is then targeted to the *trans*-Golgi network (TGN) (Hiller and Weber, 1985; Schmelz *et al.,* 1994) where it buds through the TGN, acquiring yet another double-layered membrane, forming IEV. IEV induces cytoskeletal rearrangement of the host cell actin network, which is polymerized from one side of the particle, propelling the IEV particle to and through the cell membrane (Cudmore *et al.,* 1995). Virions that remain associated with the extracellular surface of cells are referred to as CEV while those expelled into the medium are referred to as EEV.

In the current study, we used the protein encoded by the F13L ORF, p37. The 37-kDa protein is the major palmitylprotein produced by VV. The protein is 372 amino acids in length and has been shown to be palmitylated on both cysteine residues in positions 185 and 186 (Grosenbach *et al.,* 1997). p37 is expressed at late times ~4 h p.i. until cell lysis and has been found to interact with the membranes of the *trans*-Golgi network (Schmelz *et al.,* 1994). When virion associated, p37 is found exclusively on the outer envelope of EEV. It has been demonstrated by the use of a F13L deletion mutant virus, vRB10, that p37 is essential for proper virus envelopment and

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[2] Abbreviations: VV, vaccinia virus; IMV, intracellular mature virus; IEV, intracellular enveloped virus; CEV, cell-associated enveloped virus; EEV, extracellular enveloped virus; p37, 37000-dalton vaccinia envelope antigen; TGN, *trans* Golgi network; ATI, vaccinia A-type inclusion protein encoded by the A25L open reading frame; PAT, palmitylprotein acyltransferase; MEM-E, Eagle's minimal essential medium; LG, L-glutamine; GS, gentamicin sulfate; FCS, fetal calf serum; [^{3}H]PA, [9,10-^{3}H]palmitic acid; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay buffer; α-p37, rabbit polyclonal antiserum to the 37000-dalton vaccinia envelope antigen; PAGE, polyacrylamide gel electrophoresis.

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release (Blasco and Moss, 1991). Recently, our lab has expanded on these finding to show that palmitylation of p37 is necessary for appropriate targeting to the *trans*-Golgi network and protein function (Grosenbach *et al.,* 1997).

Several of the VV acylproteins have been shown to play important roles in the VV life cycle. Five VV myristylproteins have been identified as the gene products of the A25L, A16L, E7R, G9R, and L1R open reading frames (Martin *et al.,* 1997). Four of these proteins contain the canonical amino-terminal myristylation motif consisting of M-G-*X*-*X*-*X*-S/T/A/C/N) (Duronio *et al.,* 1991), where *X* is any amino acid and glycine in the second position serves as the modification site. A25L, which encodes the A-type inclusion (ATI) protein, is not myristylated at the N terminus but is internally modified possibly on an arginine or lysine residue as described previously (Martin, 1997). There are at least six VV palmitylproteins. Three species are derived from unidentified ORFs and the remaining are encoded by the A33R (Roper *et al.,* 1996), B5R (Isaacs *et al.,* 1992), and F13L (Hirt *et al.,* 1986) open reading frames.

Palmitylation is a reversible process that involves the covalent attachment of a 16-carbon saturated fatty acid via a thioester or ester bond to a cysteine or serine/threonine residue respectively (Hruby and Franke, 1993). The palmitylation of proteins facilitates one of three known functions: increasing specific protein–protein interactions, targeting proteins to membranes, and structural activation of proteins (Shenoy-Scaria *et al.,* 1994; Wedegaertner *et al.,* 1995). Unlike myristylation, little is known about the enzymology of palmitylation. It is likely that several palmityltransferases exist due to the wide diversity of protein sequences that are palmitylated. Currently there are two palmityltransferases activities known: a membrane-associated acyltransferase that is capable of palmitylating mucin glycoproteins (Kasinathan *et al.,* 1990) and another membrane-bound palmitylprotein acyltransferase (PAT) that transfers palmitate to members of the *Src* protein family (Berthiaume and Resh, 1995), and Gα subunits (Dunphy *et al.,* 1996).

Many of the known viral membrane-bound palmitylproteins are essential for viral replication or assembly (Schmidt and Burns, 1989; Hruby and Franke, 1993; Ponimaskin and Schmidt, 1995; Resh, 1996; Veit *et al.,* 1996; Grosenbach *et al.,* 1997). In each case the palmityl moiety is necessary for some aspect of protein function. With palmitylproteins playing such critical roles in many viral replication cycles, it has been of interest to predict potential palmitylproteins and their site of modification as a means to identify important viral gene products. This was first done by examining the contextual constraints for palmitylation, which includes two criteria: cysteine residues no more than 10 amino acids away from the transmembrane domain can serve as the acceptor site for palmitylation, and the palmitylation of cysteines within a transmembrane domain can only occur if located within six amino acids of the cytoplasmic borders (Ponimaskin and Schmidt, 1995). It was also realized that cysteines are not enough to direct the palmitylation of a protein, as most cysteine-containing proteins are not modified. Recently, palmitylproteins have been classified into four types (Resh, 1996). These classes are based on the site of modification, whether they are multiply modified by myristylation, palmitylation, and/or isoprenylation, and whether they interact with membranes. Type 1 palmitylproteins include transmembrane proteins that are palmitylated on cysteines close to a transmembrane spanning region. Type 2 palmitylproteins are palmitylated at the carboxylterminus and requires prior prenylation of a cysteine within a CAAX box motif. Type 3 palmitylproteins are palmitylated multiple times within the first 10–20 amino acids. Last, Type 4 palmitylproteins are both myristylated and palmitylated within the amino-terminal motif: M-G-C, in which the glycine in position two serves as the myristylation site and the cysteine in position three serves as the palmitylation site. In this latter case, palmitylation is dependent on prior myristylation.

From these classifications, it would appear that the loosely conserved motif defined by TMDX_{1-12}*AA*C(C)*A* would only predict Type 1 palmitylproteins (Grosenbach *et al.,* 1997). However, p37 does not fit any of the above classifications (p37 is not a transmembrane protein), but the motif was useful in identifying the correct site of palmitylation. This suggests that the TMD portion of the motif may only represent a region of hydrophobic sequence that may or may not be a transmembrane domain, or in the case of a dually modified protein, another hydrophobic molecule (myristate, palmitate, or prenyl group). Thus a motif that can predict the palmitylation site of many palmitylproteins can be defined as Hydro**AA*C(C)*A* (where Hydro* represents a hydrophobic portion of a protein determined by any one of the following: a hydrophobic sequence, a transmembrane domain 1 to 12 amino acids away from the modification site, or the prior addition of a hydrophobic molecule). To determine the validity of the motif site, directed mutagenesis was performed on the F13L ORF at positions upstream and downstream of the palmitate acceptor sites. Amino acids located within the motif were mutated to different residues to determine the site occupancy constraints and the requirements for a hydrophobic region to direct palmitylation. The results presented here suggest that while any amino acid within the motif is capable of directing palmitylation, residues that have hydrophobic properties are most efficient. Also, the hydrophobic region upstream of the p37 palmitylation

TABLE 1

Identification of the Palmitylation Consensus Motif

Palmitylated proteins		References
FLU-HA	MGLVFIC*V KNGNMRC*TIC*I	Veit *et al.* (1991); Veit *et al.* (1996)
VSV-G	HGLFLVL RVGIHLC*IKLKATKKQI	Rose *et al.* (1984); Veit *et al.* (1996)
CD4	GIFFC*V RC*RHRRRQAERMSQIKR	Crise and Rose (1992); Veit *et al.* (1996)
Rhodopsin	VIYIMMN KQFRNCMVTTLC*C*GKNPLGDDEA	Ovchinnikov Yu *et al.* (1988); Veit *et al.* (1996)
Trans.-rec	YC*ISGSC* RKPKTVNAKTNNDANEEEDVALKM	Omary and Trowbridge (1981); Veit *et al.* (1996)
Marbur virus GP	LSC*IC* RIFTKYIG	Volchkov *et al.* (1998); Veit *et al.* (1996)
F-MuLV env	LFGPC*ILN RLVQFVKDRISVVQAL	Hensel *et al.* (1995); Veit *et al.* (1996)
HEF/HA chim	GIAIC*V KNGNMRC*TIC*I	Veit *et al.* (1990); Veit *et al.* (1996)
Sindbis E2	TVAVLC*AC* KARREC*LTPYALAPNAVIPTSLALLC*C*VRSA	Ivanova and Schlesinger (1993); Veit *et al.* (1996)
SFV E2	SC*YMLVAA RSKC*LTPYALTPGAAVPWTLGILC*C*APRAH	Ivanova and Schlesinger (1993); Veit *et al.* (1996)
AdenoDP	FVCLIIWL IC*C*LKRK	Hausmann *et al.* (1998)
Hum B2AR	TMD(−5) IAFQELLC*LRRSSLKAYG	O. Dowd *et al.* (1989)
Hum B1R	TMD(−6) KAFQGLLC*C*ARRAARRRH	Frielle *et al.* (1987)
Hum α1AR	TMD(−7) AFMRILGC*QCRSGRRRRR	Schwinn *et al.* (1995)
Lyk	MGC*VCSSNPED	Shenoy-Scaria *et al.* (1993)
BovineNOS3	GPPC*GLGLGLGLGLC*GKQGPAS	Robinson and Michel (1995)
band III	FTGIQIIC*LAVLWVVKST	Okubo *et al.* (1991)
RasK	KEEKTPGC*VKIKKCIIM	Barbacid (1987)
Mouse SSR2	KKSFQNVLC*LVKVSGT	Yamada *et al.* (1992)
p37 (IHD-J)	LNLCSAAC*C*LPVSTAYHIK	Grosenbach *et al.* (1997)
Consensus	TMDX_{1-12}*AA**C(C)**A* Hydro**AA**C(C)**A*	Grosenbach *et al.* (1997)

Note. The computer program Nentrez was used to compile the protein sequences of numerous known palmitylproteins. For each palmitylprotein analyzed, the site of modification and various protein properties were determined. This information allowed each protein to be aligned with the site of modification serving as the reference point. Similarities in sequence were determined by physical properties and mathematically by percent occupancy for each residue individually and then by amino acid class. From this analysis, the consensus motif, TMDX_{1-12}*AA*C(C)*A* was identified. Comparing the motif to p37's amino acid sequence allowed the determination of the site of palmitylation. The site of modification corresponds to the amino acids in positions 185 and 186, with aliphatic residues occupying positions 183, 184, and 187. C* represents putative or actual cysteine modification sites. Gapped sequences represent the putative boundary between a transmembrane domain and the cytoplasmic tail of the protein. The above motif has since been changed to Hydro**AA*C(C)*A,* so that it can be used to predict palmitylproteins of different types (types 1–4).

site is absolutely required for protein palmitylation. The motif Hydro**AA*C(C)*A,* may be useful in predicting the modification site of most palmitylproteins.

RESULTS

Analysis and alignment of palmitylproteins

The amino acid sequence of p37 was deduced from the nucleotide sequence of the VV strain IHD-J F13L gene (Blasco and Moss, 1991). Grosenbach and Hruby (Grosenbach *et al.,* 1997) have previously demonstrated that the cysteines in positions 185 and 186 of p37 serve as the palmitate acceptor sites. Computer-assisted analysis of p37 was used to predict protein hydrophobicity and putative transmembrane spanning domains and protein secondary structure. Hydrophobicity plots revealed that the central portion of p37 contains two hydrophobic regions (data not shown). The region consisting of the amino acids from positions 172–198 (encompassing the palmitylation sites) is predicted to be a transmembrane domain. Secondary structure predictions showed p37 to consist of 36% α helix, 30% β sheet, 21% turns, and the remaining 13% other structures.

Ponamaskin and Schmidt (1995) have previously described criteria for palmitylation of viral glycoproteins. However, using this criteria we could not predict the modification site of p37. To facilitate the identification of the modified residues in p37, numerous palmitylproteins were examined of which the sequence and site(s) of modification had already been determined. Each known palmitylprotein was subjected to the same type of analysis that p37 was subjected to, considering secondary structures and membrane topologies (data not shown). An alignment was made in which the sequence of each palmitylprotein was aligned using the site(s) of modification as the reference point (Table 1). Primary structural properties were determined for each protein considering specific amino acid, amino acid properties, and amino acid classes. Based on this data, it appeared that palmitylation occurs most frequently 1–12 amino acids downstream (on the cytoplasmic side) of a transmembrane spanning region, downstream of a hydrophobic sequence, or immediately following the modification of the protein by another fatty acid. Also the modified cysteines are preceded by two aliphatic residues and followed by

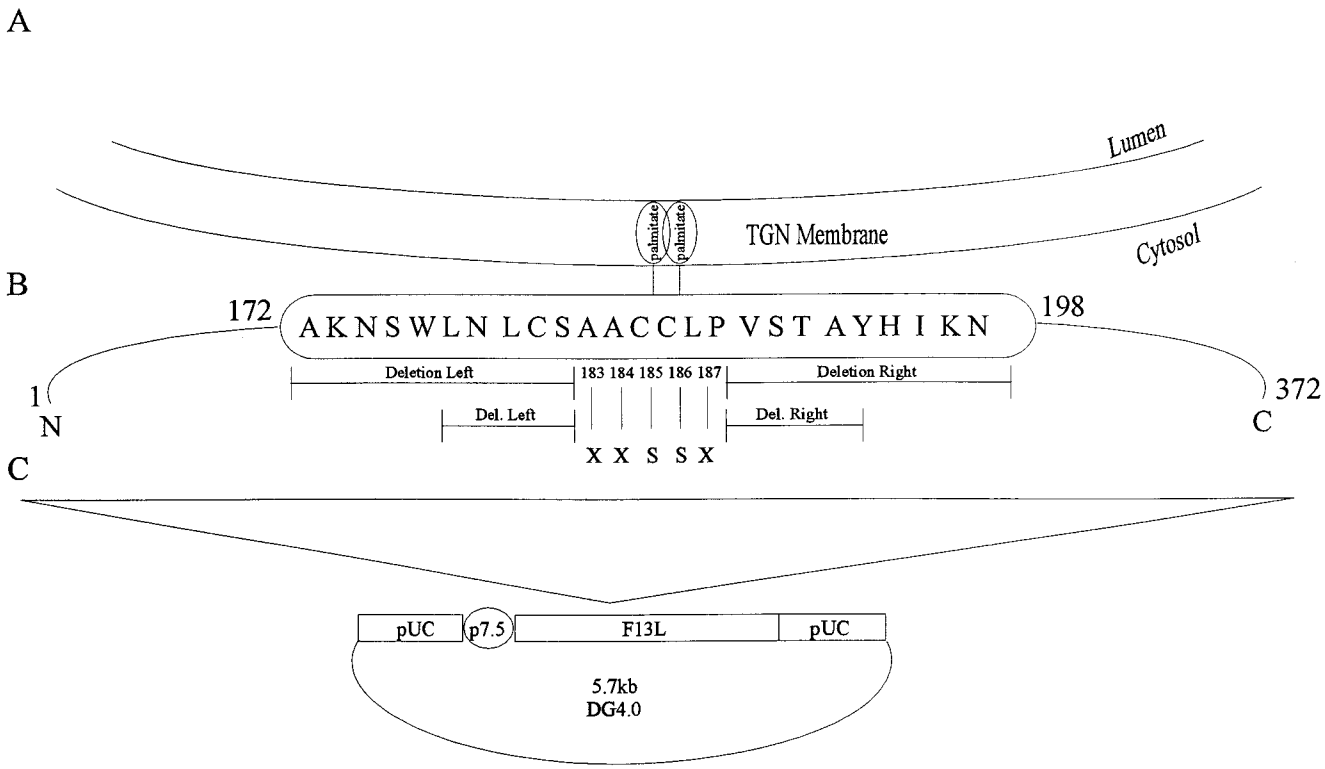

FIG. 1. Design and construction of the transient expression vector DG4.0 and mutated derivatives. (A) Diagram of the hypothetical p37: membrane interaction mediated by its palmityl moieties. (B) The hydrophobic region of p37 from residues 172–198 are depicted using the single-letter amino acid code. The wild-type amino acids in position 183, 184, and 187 were targeted for mutagenesis. (C) An 1110-bp DNA fragment containing the wild-type VV F13L open reading frame was inserted downstream of the 7.5 early/late promoter to allow for constitutive expression throughout the viral infection.

another aliphatic residue. This motif was thus defined as $TMDX_{1-12}AAC(C)A$, where TMD is a transmembrane domain, *X* is any amino acid, *A* is any aliphatic amino acid, and C is the palmitylated cysteine(s). This motif enabled the identification of the palmitylation site in p37, in which two alanines precede the cysteine doublet in positions 185 and 186, which is then followed by a leucine. However, the modification site of p37 is predicted to be within a transmembrane spanning domain, which does not agree structurally with the predicted motif. However, Ponamaskin and Schmidt have also reported that palmitylation of cysteines can occur within a transmembrane domain. In any case, the motif had enabled the identification of the palmitylation sites of p37. To this end, it was of interest to determine the requirements for a hydrophobic region, the amino acid site occupancy constraints, and if the motif could be expanded to include palmitylproteins of different types.

Plasmid construction and oligonucleotide-directed mutagenesis

A F13L deletion mutant virus, vRB10, has had 93% of the F13L open reading frame deleted by insertional inactivation using an expression cassette mediating mycophenolic acid resistance. Even though vRB10 does not express p37, it is still viable in tissue culture, but the virus is unable to efficiently form plaques on susceptible monolayers of cells as a result of producing very little CEV or EEV. Reinsertion of the F13L gene into the vRB10 genome or transient expression of p37 in vRB10-infected cells restores the wild-type plaque-forming phenotype, confirming the essentiality of p37 to this process (Grosenbach *et al.,* 1997).

Use of the transient expression vector pDG4.0 enabled the expression of both wild-type and mutant forms of p37. A nonpalmitylated form of p37 was also constructed in which the cysteine doublet had been mutated to a serine doublet, and two other mutants, which are less efficient at incorporating palmitate due to a mutation of one of the cysteines to a serine (pCC, pSC, and pCS, respectively). To build on these earlier findings, oligo-directed mutagenesis was used to mutate amino acids at positions within the consensus motif and within the predicted hydrophobic region (Fig. 1). By the use of three separate degenerate oligonucleotide primers, amino acids in positions 183, 184, and 187 of p37 were mutated to different amino acids. The goal for each position was to obtain

mutants that represented the different classes of amino acids.

Computer predictions indicate that there are 10 hydrophobic amino acids upstream and downstream of the palmitylation site (positions 185/186). To determine the requirement for the hydrophobic sequence surrounding the palmitate acceptor site of p37, four deletion mutants were constructed. Two deletion mutants deleted all or half of the 10 amino acids upstream of the palmitylation site (LΔ10 and LΔ5, respectively), while the other two deletion mutants eliminated all or half of the ten amino acids downstream of the palmitylation site (RΔ10 and RΔ5, respectively). In position 183 the wild-type alanine was changed to glycine (A183G), valine (A183V), serine (A183S), threonine (A183T), proline (A183P), tryptophan (A183W), lysine (A183K), and aspartic acid (A183D). The alanine is position 184 was changed to valine (A184V), serine (A184S), threonine (A184T), proline (A184P), phenylalanine (A184F), tyrosine (A184Y), tryptophan (A184W), lysine (A184K), and aspartic acid (A184D). In position 187, the wild-type leucine was mutated to glycine (L187G), valine (L187V), serine (L187S), cysteine (L187C), threonine (L187T), methionine (L187M), proline (L187P), phenylalanine (L187F), tryptophan (L187W), lysine (L187K), and glutamic acid (L187E). These mutants made used to determine the site occupancy constraints upstream and downstream of the palmitate acceptor site.

Analysis of palmitate incorporation/efficiency

Confluent monolayers of cells were infected with the F13L deletion mutant, vRB10, and then transfected with a wild-type p37 transient expression vector (DG4.0), the mutant p37 transient expression vectors for positions 183, 184, 187, and the deletion mutants. Transfected cell extracts were harvested and processed to first measure the expression of p37 and second for modification of p37. The samples were treated with reducing sample buffer, which may cleave the thioester bond between p37 and palmitate, but all samples were treated identically and were analyzed immediately. To quantitate protein expression, gel electrophoresis together with chemiluminescence procedures were employed (Figs. 2a, 3a, and 4a). Protein production was detected for each transiently expressed mutant gene. An equivalent fraction of the same total cell extracts were examined next for incorporation of [^{3}H]-palmitic acid into p37 using gel electrophoresis followed by fluorography (Figs. 2b, 3b, and 4b). Mutations in positions 183, 184, and 187 did not block palmitylation of p37, however, the majority of the mutated proteins were less efficient at incorporating label. For each position within the motif, palmitylation of p37 could be detected, while only three of the four deletion mutants retained activity. Film densitometry was used to quantify the amount of signal obtained on the gels subjected to fluorography and chemiluminescence. Densitometry has been used previously to quantify relative amounts of protein and signal (Hancock *et al.,* 1991). The amounts determined through film densitometry were used to obtain a ratio of protein production to [^{3}H]-palmitate incorporation (data not shown). The wild-type expression from plasmid DG4.0 was used as the standard, and the percent for the mutants were determined based on this value (Figs. 2c, 3c, and 4c). Three other control plasmids were used in these experiments pCS, pSC, and pSS. Plasmids pCS, pSC, and pSS are DG4.0 derivatives that have had one or both cysteines at positions 185 and 186 (palmitate acceptor sites in p37) mutated to serine. Previous transfection experiments with these plasmids have demonstrated that palmitylation is abolished with the double cysteine mutant (pSS) and less efficient with the single cysteine mutants (pCS and pSC). A minor palmitylprotein with an apparent molecular weight of 37 kDa is still observed in pSS lanes (Grosenbach *et al.,* 1997). The same palmitylprotein can be seen in vRB10-infected cells and does not produce any p37. The protein observed in this position was considered background and subtracted away from the densitometry readings (value set to 0% incorporation of palmitate). Most of the mutants tested were less efficient at incorporating label; however, the results do indicate preferences for different types of amino acid. The palmitylation efficiency for the four deletion mutants was significantly less than DG4.0. Palmitylation was not observed when the entire 10 upstream amino acid sequence was deleted from p37 (LΔ10). Whereas, LΔ5 (42%), RΔ10 (26%), and RΔ5 (31%) still incorporated label but less efficiently (Fig. 2c). In position 183, the results show a preference for the wild-type alanine. In general, hydrophobic residues or residues with a small side chain (glycine) in this position were more efficient at incorporating label as seen with A183G (47%), A183V (43%), A183P (43%), and A183W (39%) compared with A183S (33%), A183T (35%), A183K (38%), and A183D (19%) (Fig. 3c). Results obtained for position 184 were similar to those observed in position 183 in that the wild-type alanine was the preferred residue. Although, the hydrophobic amino acids represented by A184P (32%), A184F (37%), A184Y (42%), and A184W (31%) were more efficient at directing palmitylation than A184S (28%), A184T (11%), A184K (22%), and A184D (5%), the mutant A184V (48%), which has an aliphatic residue, was the most efficient. In position 187, the mutants L187G (120%), L187V (93%), L187S (69%), L187C (89%), L187T (112%), L187M (116%), L187F (72%), and L187E (67%) allowed for incorporation of label equal to, or in slightly greater amounts than, the wild-type leucine. In comparison, the mutants L187P (11%), L187W (28%), and L187K (44%) were not preferred in position 187.

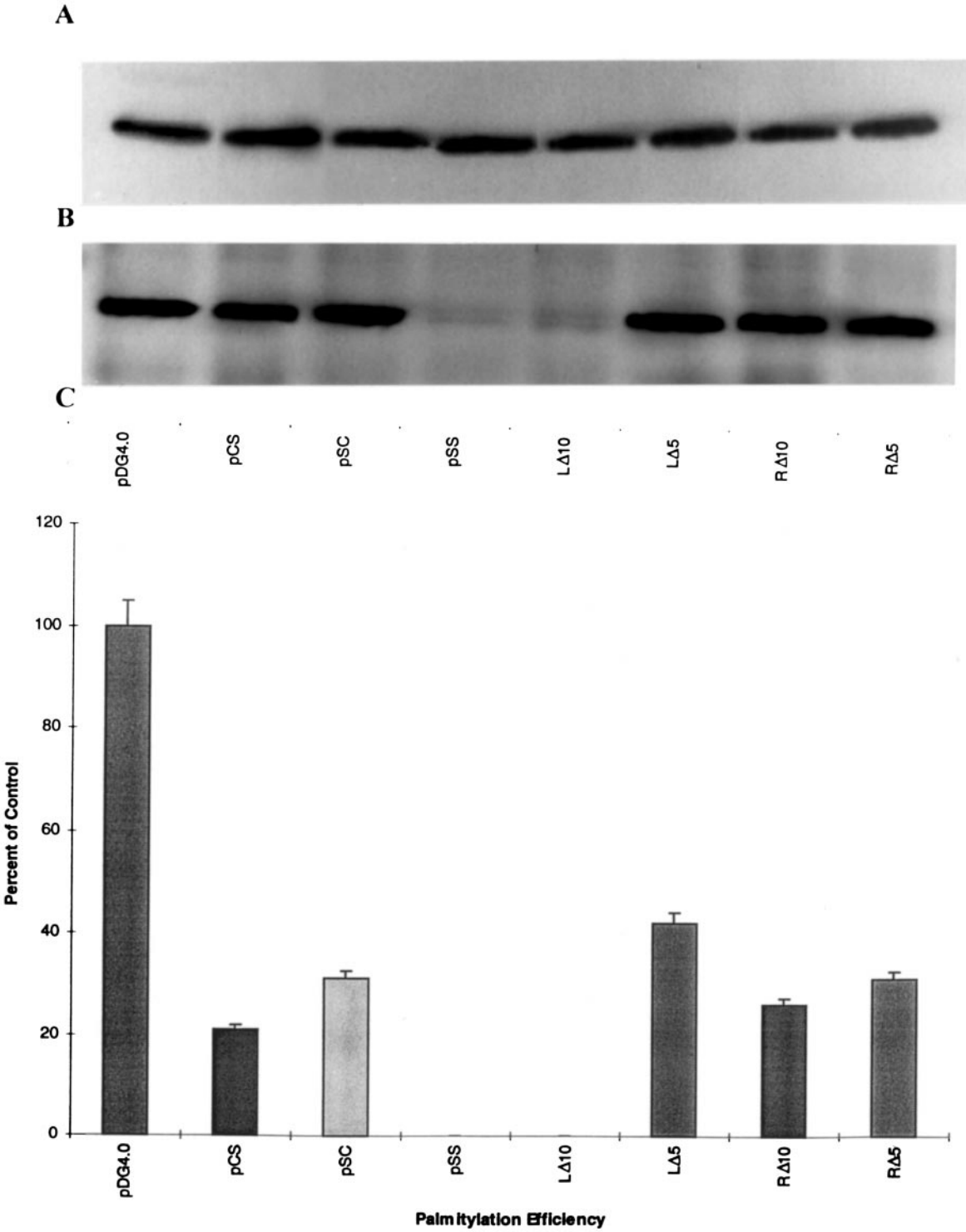

FIG. 2. Analysis of palmitylation efficiency for transmembrane deletion mutants. BSC_{40} cells were infected with vRB10 and then transfected both with the wild-type p37 transient expression vector (DG4.0) and mutated versions of this plasmid. Tritiated palmitic acid was added to the culture medium after 6 h p.i. Total cell extracts were harvested at 24 h p.i. (A) A fraction of the extract was subjected to SDS–PAGE and chemiluminescence using anti-p37 as the primary antibody. (B) An equivalent amount of total cell extract was subjected to SDS–PAGE and fluorography to detect incorporation of label, a measure of palmitylation. (C) Protein-antibody complexes were quantitated by application of chemiluminescent substrate and exposure to film followed by film densitometry. The fluorograph of palmitylation was also quantified by film densitometry. The percent of incorporation of label *vs* protein production is depicted by the bar graph. Results were determined from an average of three separate experiments.

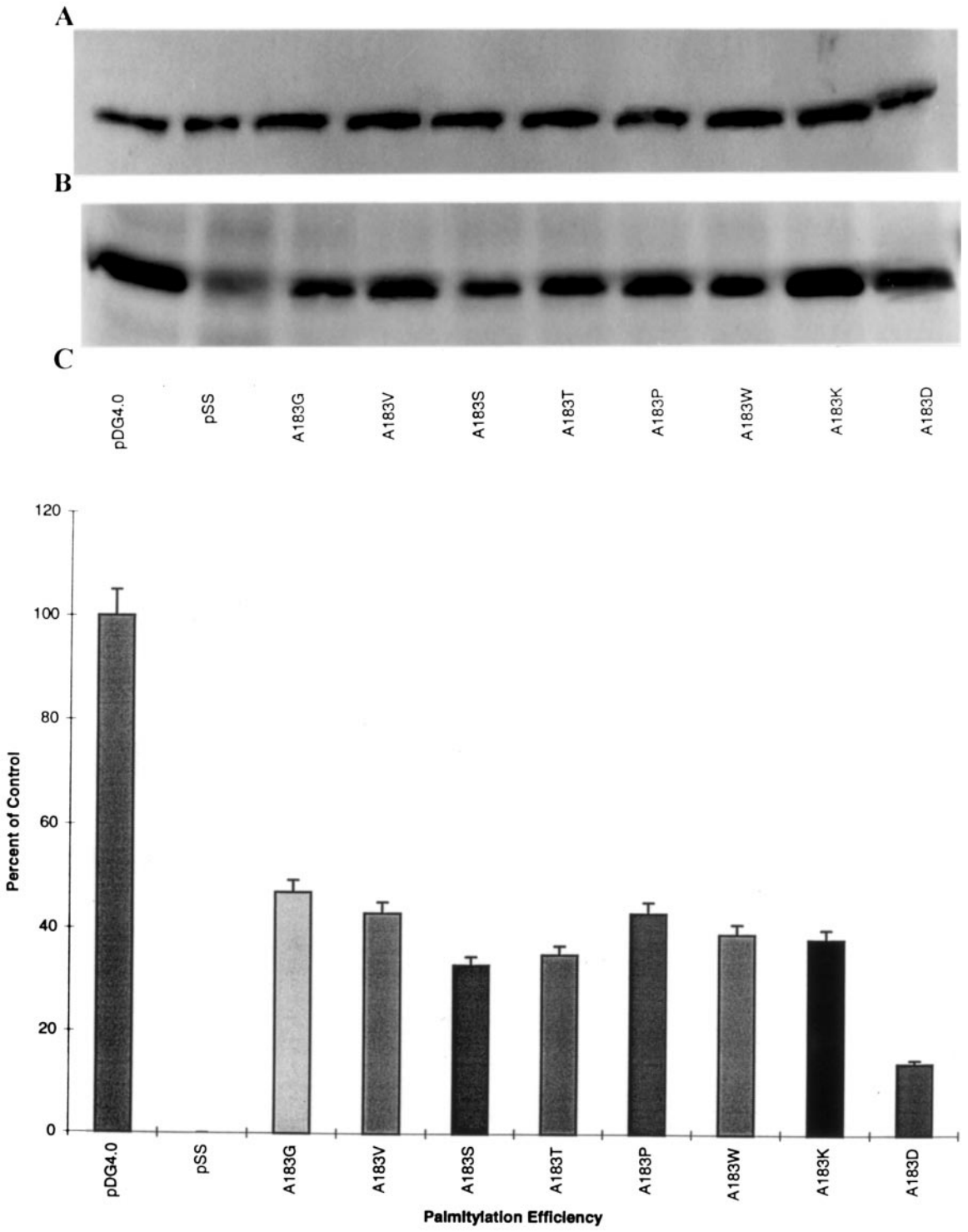

FIG. 3. Analysis of palmitylation efficiency for position 183. BSC_{40} cells were infected with vRB10 and then transfected both with the wild-type p37 transient expression vector (DG4.0) and mutated versions of this plasmid. Tritiated palmitic acid was added to the culture medium after 6 h p.i. Total cell extracts were harvested at 24 h p.i. (A) A fraction of the extract was subjected to SDS–PAGE and chemiluminescence using anti-p37 as the primary antibody. (B) An equivalent amount of total cell extract was subjected to SDS–PAGE and fluorography to detect incorporation of label, a measure of palmitylation. (C) Protein-antibody complexes were quantitated by application of chemiluminescent substrate and exposure to film followed by film densitometry. The fluorograph of palmitylation was also quantified by film densitometry. The percent of incorporation of label *vs* protein production is depicted by the bar graph. Results were determined from an average of three separate experiments.

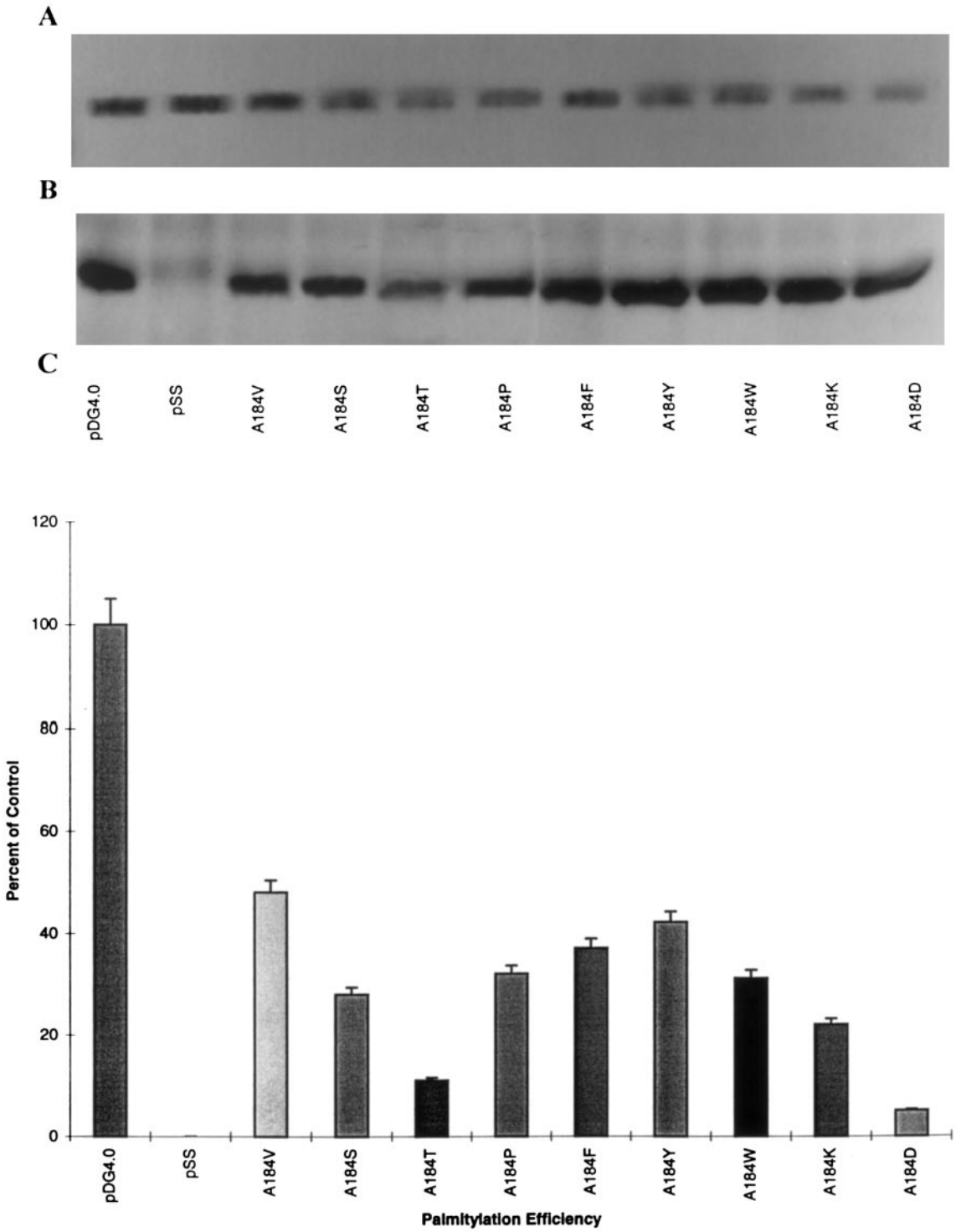

FIG. 4. Analysis of palmitylation efficiency for position 184. BSC_{40} cells were infected with vRB10, and then transfected both with the wild-type p37 transient expression vector (DG4.0) and mutated versions of this plasmid. Tritiated palmitic acid was added to the culture medium after 6 h p.i. Total cell extracts were harvested at 24 h p.i. (A) A fraction of the extract was subjected to SDS–PAGE and chemiluminescence using anti-p37 as the primary antibody. (B) An equivalent amount of total cell extract was subjected to SDS–PAGE and fluorography to detect incorporation of label, a measure of palmitylation. (C) Protein-antibody complexes were quantitated by application of chemiluminescent substrate and exposure to film followed by film densitometry. The fluorograph of palmitylation was also quantified by film densitometry. The percent of incorporation of label *vs* protein production is depicted by the bar graph. Results were determined from an average of three separate experiments.

DISCUSSION

By analysis of numerous palmitylproteins, a loosely conserved palmitylation motif, TMD$X_{1-12}AAC$(C)A, in which the palmitylated cysteines (C) are preceded by two aliphatic residues (*A*), and followed by another was identified (Grosenbach *et al.,* 1997). Several proteins studied were palmitylated within or proximal to a transmembrane domain (TMD). This motif is based on criteria that have been observed for palmitylated viral glycoproteins and numerous cellular palmitylproteins (Schmidt and Burns, 1989). This motif aided in the identification of the palmitylation site in the VV 37-kDa envelope protein, p37 (Grosenbach *et al.,* 1997). In this report, the validity of this motif was examined by testing the requirements for a transmembrane spanning domain and determining what amino acid occupancy restrictions exist, if any, in the residues that immediately preceding or following the palmitylated cysteines. To study the components of the motif, a derivative of the transient expression vector pDG4.0, which has the intact F13L gene behind the VV 7.5k promoter, was used. Use of a VV F13L deletion mutant, vRB10 which does not express p37, together with pDG4.0 derivatives enabled the examination by transient expression of p37 and palmitylation efficiency.

Significance of the hydrophobic domain surrounding the palmitylation site

Chou–Fasman and Hopp–Woods analyses predict that p37 contains two hydrophobic domains, one of which is predicted to be a transmembrane spanning domain. However, there are data to indicate that p37 does not span the membrane. Detergent partitioning studies were done using native fully palmitylated p37 and p37 treated with hydroxylamine, which strips away the acyl-group by hydrolyzing the labile thioester bond between palmitate and p37. For the treated sample in which there was no palmitate attached to p37, it was found to partition to the aqueous phase (Schmutz *et al.,* 1995). These results were later confirmed *in vivo* by differential centrifugation subcellular fractionation and immunofluorescent microscopy on both wild-type and nonpalmitylated mutants of p37 (Grosenbach *et al.,* 1997). This suggests that the palmitate moiety mediates membrane interaction, much as has been observed for the human immunodeficiency virus and simian immunodeficiency virus gp41 protein (Yang *et al.,* 1995). It is postulated that a palmitylated gp41 protein stabilizes the interaction of an amphipathic region with the membrane.

To test whether the hydrophobic sequence surrounding the palmitylation site affected palmitylation, four deletion mutants were constructed, only one of which completely eliminated palmitylation. The data suggest that the amino acids preceding the palmitylation site were necessary for palmitylation. The three other deletion mutants affected palmitylation by reducing the efficiency of label incorporation by more than half in some cases. The reason for this is unknown, but possibly the deletion interfered with the secondary structure or hydrophobic properties of p37, making it difficult or impossible for the protein to associate with the membrane and thus preventing palmitylation. The results obtained in these experiments together with the alignments presented in Table 1 would suggest that the requirement for the upstream sequence is not sequence specific but property (hydrophobic and secondary structure) specific. This specificity is seen with all types of palmitylproteins. Different hydrophobic/secondary structure elements are present depending on which type of palmitylprotein one is studying but are generally one of the following: a hydrophobic sequence, a transmembrane domain 1–12 upstream of the palmitate acceptor site, or another hydrophobic moiety such as myristate. It can be concluded that the TMD portion of the motif is not required for palmitylation rather a hydrophobic element is needed or a specific secondary structure of the protein.

Significance of residues contained within the consensus motif

From the above conclusion one would expect that hydrophobic amino acids would perform better within the consensus motif. To test this hypothesis, numerous mutants were constructed for each site within the motif using a degenerative oligonucleotide with intention of obtaining at least one residue from each amino acid class (Table 2). Position 183 was analyzed first by testing several amino acids in this position and comparing their palmitylation efficiency to that of the wild-type protein (Fig. 3). For all amino acids analyzed, palmitylation efficiency was reduced by >50% and dropped to as low as 20% when aspartic acid was in this position. As expected the hydrophobic amino acids were more efficient in directing palmitylation. The aliphatic residue glycine in position 183 gave the best results, although efficiency was significantly less than the wild-type alanine. These results suggest a preference for amino acids with hydrophobic properties, or for amino acids with small side chains (glycine). Position 184, the second aliphatic amino acid in the motif, was analyzed next. Numerous amino acids were tested in this position and again the preferred residue was the wild-type alanine (Fig. 4). However, this time the graph revealed a different overall activity profile. Although, palmitylation was significantly reduced with every residue, the mutants with >30% efficiency were amino acids with hydrophobic properties. Again these results suggest a preference for hydrophobic amino acids with aliphatic residues giving the best results. The results obtained by mutating position 187, the third aliphatic residue within the motif, were quite different. The

TABLE 2

Summary of the TMDX_{1-12}*AAC*(C)*A* Mutants

Mutants	Amino acid property	Sequence changes	Palmitylation efficiency (percent of wild type)
A183G	Aliphatic	Small side chain	47
A183V	Aliphatic	Hydrophobic amino acid	43
A183P	Cyclic	Hydrophobic amino acid	43
A183W	Aromatic	Hydrophobic amino acid	39
A183S	Hydoxl/sulfur	Polar	33
A183T	Hydroxl/sulfur	Polar	35
A183K	Basic	Charged	38
A183D	Acidic	Charged	19
A184V	Aliphatic	Hydrophobic	48
A184P	Cyclic	Hydrophobic	32
A184F	Aromatic	Hydrophobic	37
A184Y	Aromatic	Hydrophobic	42
A184W	Aromatic	Hydrophobic	31
A184S	Hydroxl/sulfur	Polar	28
A184T	Hydroxl/sulfur	Polar	11
A184K	Basic	Charged	22
A184D	Acidic	Charged	5
L187G	Aliphatic	Small side chain	120
L187V	Aliphatic	Hydrophobic	93
L187S	Hydroxl/sulfur	Polar	69
L187C	Hydroxl/sulfur	Polar	89
L187T	Hydroxl/sulfur	Polar	112
L187M	Hydroxl/sulfur	Polar	116
L187F	Hydroxl/sulfur	Hydrophobic	72
L187E	Acid	Charged	44

Note. The table represents the data obtained by mutating the amino acids in positions 183, 184, and 187 of p37. Mutants have been defined in the text and in Figs. 2–5.

site occupancy for position 187 was considerably less stringent compared to the other positions analyzed (Fig. 5). Several amino acids with varying properties (hydroxyl-sulfur containing, acidic, aromatic, and basic) allowed for palmitylation efficiency comparable to that of the wild-type leucine. Surprisingly, a glycine in position 187 seemed to drastically increase palmitylation efficiency for reasons which are unknown. The only residue that gave poor efficiency of palmitylation was proline, possibly as a result of an unfavorable change in secondary structure. The results obtained for 187 suggest that essentially any amino acid can be placed in this position except proline.

From these results, several conclusions can be reached: (i) The aliphatic amino acids preceding the palmitylation site are required for efficient palmitylation of p37. (ii) Alanine is the preferred residue in positions 183 and 184. (iii) Site occupancy in position 187 is permissive. (iv) Last the motif can be defined as Hydro**AAC*(C)*A* (where Hydro* represents a hydrophobic portion of a protein determined by any one of the following: a hydrophobic sequence, a transmembrane domain 1–12 amino acids away from the modification site, or the prior addition of a hydrophobic molecule; C, palmitate acceptor cysteines; *A*, aliphatic residue). Although, the aliphatic alanines were the preferred residues preceding the palmitate acceptor site, another aliphatic residue following the cysteines actually enhances modification.

These results suggest that the motif may require further refinement to predict the palmitylation sites in other proteins. However, the motif was sufficient in predicting the correct palmitylation site for p37. Other VV palmityl-proteins will be analyzed using this motif to test its utility in predicting modification sites. VV has proved to be a useful tool in the study and analysis of eukaryotic protein modification and processing. Continued studies of VV will hopefully lead to a better understanding of the many processes that govern other viral systems and, more importantly, give us new insights into protein palmitylation.

MATERIALS AND METHODS

Cells and virus

African green monkey kidney cells (BSC_{40}) were grown and maintained in modified Eagle's minimal essential medium (MEM-E, Sigma) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Summit Biotechnology), 2 mM L-glutamine (LG), 10 μg/ml gentamicin

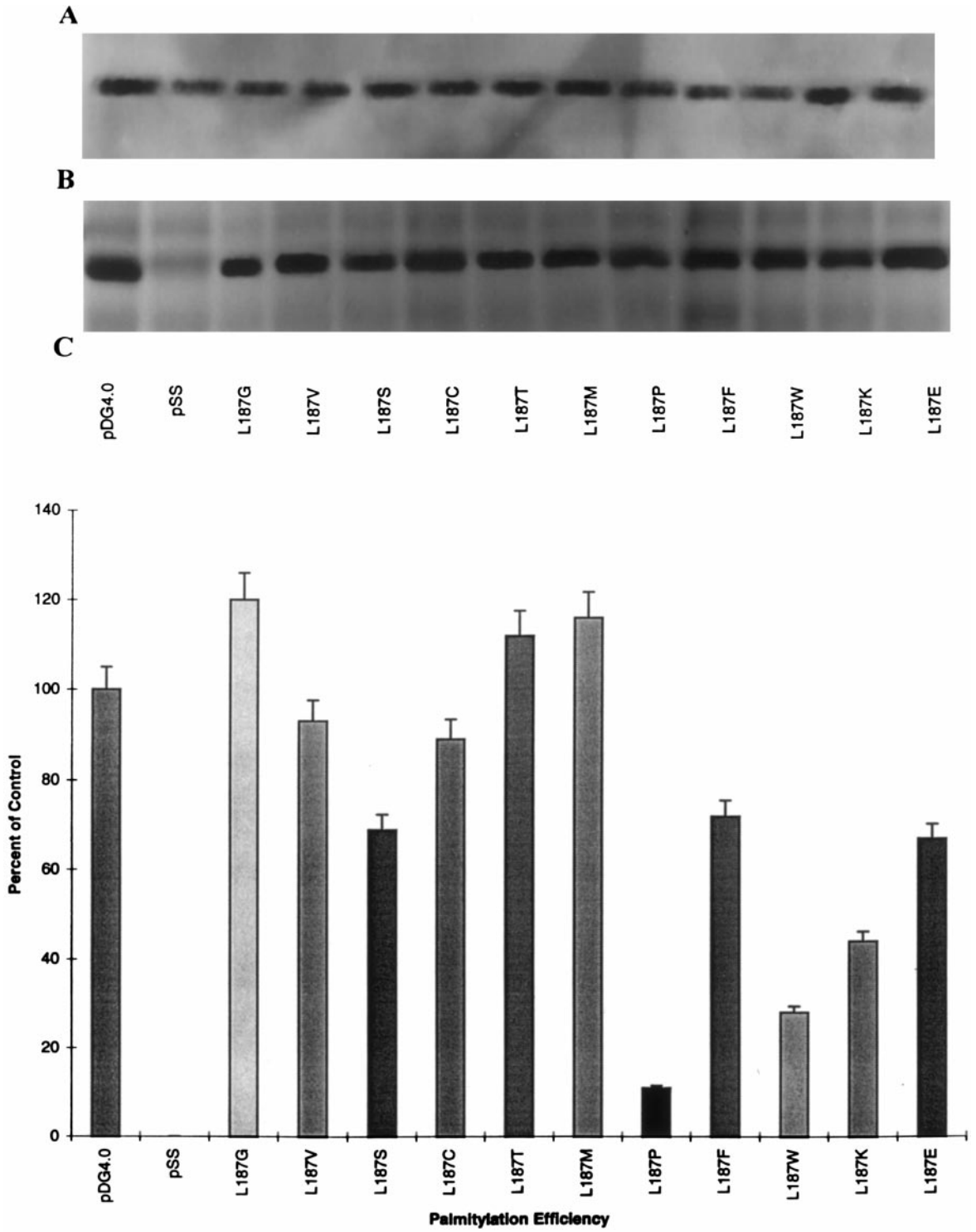

FIG. 5. Analysis of palmitylation efficiency for position 187. BSC_{40} cells were infected with vRB10 and then transfected both with the wild-type p37 transient expression vector (DG4.0) and mutated versions of this plasmid. Tritiated palmitic acid was added to the culture medium after 6 h p.i. Total cell extracts were harvested at 24 h p.i. (A) A fraction of the extract was subjected to SDS–PAGE and chemiluminescence using anti-p37 as the primary antibody. (B) An equivalent amount of total cell extract was subjected to SDS–PAGE and fluorography to detect incorporation of label, a measure of palmitylation. (C) Protein-antibody complexes were quantitated by application of chemiluminescent substrate and exposure to film followed by film densitometry. The fluorograph of palmitylation was also quantified by film densitometry. The percent of incorporation of label *vs* protein production is depicted by the bar graph. Results were determined from an average of three separate experiments.

sulfate (GS), at 37°C, 95% humidity, and 5% CO_2. The VV IHD-J strain was grown and titered by plaque assay in BSC_{40} cells as described previously (Blasco and Moss, 1991). The vRB10 strain, also described previously, was grown using a low multiplicity of infection in BSC_{40} cells in the presence of mycophenolic acid, xanthine, and hypoxanthine. Since vRB10 has 93% of the F13L ORF deleted, it produces minute plaques, so titers were obtained by inoculating serial dilutions of virus onto a confluent monolayer of BSC_{40} cells followed by transfection of a plasmid born rescuing copy of the F13L gene. After 72 h p.i., the infected monolayer was stained with crystal violet to visualize plaques.

Plasmid construction

The p37 transient expression vector pDG4.0, described previously (Grosenbach *et al.,* 1997), is derived from the pUC118 cloning vector in which the F13L ORF is inserted downstream of a VV 7.5K promoter directing constitutive expression of p37. The Kunkel method of oligonucleotide (oligo)-directed mutagenesis (Kunkel *et al.,* 1991) was used to mutate the F13L gene within pDG4.0 to generate multiple amino acid mutants in positions 183, 184, and 187 of p37, immediately preceding and following the palmitate-acceptor cysteines. The following oligo was used to generate the degenerative mutants in position 183 while introducing an unique restriction site *Sph*I: 5′ CTGGCAGACAGCATGCNNNAGAGCATAAATTCAACC 3′, where NNN is any nucleotide. For position 184 the degenerate oligo 5′ GTGCTAACCGGTAGACAACANN NCGCAG 3′ was able to introduce the unique restriction site *Age*I. The oligo 5′ GCTAACTGGNNNACAACAAGCCGCGGAGC 3′ introduced a degeneracy in position 187 while introducing a *Sac*II site. Four transmembrane deletion mutants were constructed: LΔ10, using oligo 5′ GGCAGACAACAAGCTTTTGCGCTGCTATTAAAG GC 3′, deleted 10 amino acids upstream of the modified cysteines and introduced an unique *Hin*dIII site. LΔ5, using oligo 5′ GGCAGACAACAAGCATTCAACCATGAATTCTTTG CGC 3′, deleted five amino acids upstream of the modified cysteines and introduced an unique *Eco*RI site. RΔ10, using oligo 5′ CTCCACCAATTGGATTCTTCAGACAACAA GCCG 3′, deleted 10 amino acids downstream of the modified cysteines and introduced an unique *Mun*I site. RΔ5, using oligo 5′ CTTAATATGATACGCCAGACAGCATGCCGC 3′, deleted five amino acids downstream of the modified cysteines and introduced an unique *Sph*I site. All mutations were confirmed by DNA sequencing.

Computer-assisted analysis and alignment of palmitylproteins

The computer program Net Entrez was used to search for and retrieve the amino acid sequences of numerous known palmitylproteins including the p37 sequence. Each sequence was then analyzed using the computer program TMPred (Hofmann and Stoffel, 1993) to identify potential transmembrane spanning domains and possible orientations in a membrane. Amino acid sequences of each protein were aligned using the palmitylated cysteines as a reference point. Amino acids were then analyzed upstream and downstream of the acceptor site for primary structural consistencies.

Transient expression and analysis of palmitate incorporation/efficiency

IHD-J and vRB10 were used to infect monolayers of cells (2.5×10^6 cells) contained within a 35 × 10 mm dish at a m.o.i. of 10. vRB10-infected cells were transfected with 10 μg of plasmid DNA using DMRIE-C reagent to enable the transient expression of both wild-type (wt) and mutated forms of p37. The inoculum was prepared by adding 1 ml of MEM-E containing LG, and GS to a polystyrene tube. Ten micrograms of DNA was added to each tube along with 25 μl of liposomes (DMRIE-C, supplied by Gibco). After 15 min at room temperature, 10^7 pfu of virus was added to each tube and then added to the monolayer of cells from which the culture media had been aspirated. The cells were then incubated at 37°C for 4 h, after which the transfection inoculum was aspirated and replaced with 1 ml of MEM-E containing 200 μCi of [^{3}H]-palmitic acid (^{3}HPA, supplied by DuPont NEN). At 24 h p.i., infected cells were harvested in the culture supernatant. Cells were pelleted by centrifugation at 15,000 *g* for 30 min at 4°C and then resuspended in 100 μl of 1× phosphate-buffered saline (PBS). The resuspended pellet was froze and then thawed three times after which 50 μl of 3× reducing sample buffer was added. The sample was then boiled for 3 min and then centrifuged at 15,000 *g* for 1 min to pellet the insoluble material. Each sample (13 μl) was loaded on two identical 12% polyacrylamide gels and resolved by discontinuous gel electrophoresis (SDS–PAGE) as described previously (Studier, 1973). After electrophoresis, one gel from each sample was impregnated with 22.2% Diphenyloxazole (PPO) in Me_2SO (DMSO), dried and then exposed to Kodak BIOMAX MR film at −70°C for fluorography. The second gel was subjected to immunoblot analysis using anti-p37 antiserum (αp37). The antigen-antibody complex was then incubated with goat anti-rabbit antiserum conjugated to horseradish peroxidase (supplied by Pierce). The blot was developed by incubation with chemiluminescent substrate and exposed to Kodak BIOMAX MR film. The fluorograph of palmitate labeled proteins as well as the film of the chemiluminescent blots were analyzed by densitometry, so that a relative amounts of protein produced and label incorporated could be determined.

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